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REVIEW ARTICLE

PROSPECTS IN NASAL VACCINATION AGAINST CLINICALLY RELEVANT PATHOGENS AND SELECT AGENTS

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SUMMARY

Intranasal immunization induces mucosal immune responses in both the respiratory system and at other distant mucosal surfaces, as well as systemic immune responses. However, most vaccines are still given via the parenteral route, and to date, the only intranasal vaccine that is available to the public in the U.S. is a live, attenuated influenza vaccine. Over the last 10 years, there have been substantial efforts to develop effective nasal vaccination strategies in animal models against several different pathogens, some being successful enough to progress to phase I clinical trials. The purpose of this review is to discuss: 1) the progress made with representative models; 2) how vaccination through the intranasal route overcomes many of the shortcomings associated with the use of parenteral vaccines; and 3) antigens that have been demonstrated to be effective vaccines when administered intranasally. This review focuses on vaccine strategies against specific pathogens that are significant public health threats, or have the potential to be used as bioweapons.

INTRODUCTION

The idea of inducing immunity against a pathogen by introducing an antigen to the host via the intranasal route is not new. Before the concept of vaccination was understood by the scientific/medical community, physicians practiced variolation, whereby immunity against smallpox was obtained by inhaling powder derived from the crushed scabs of individuals infected with the smallpox virus. In 1796, Edward Jenner then discovered that the introduction of vaccinia virus through the skin could also protect individuals from developing the disease, and that this was much safer than variolation (1). Variolation, however, demonstrated that the introduction of antigen to the respiratory system via the intranasal route could generate protective immune responses against a highly virulent disease.

Currently, vaccine use is widespread in both humans and livestock. Vaccines offer a cost-effective means to prevent many diseases, including those caused by pathogens that are becoming increasingly antibiotic-resistant. However, few vaccines developed for human use are given via the intranasal route. Thus, during the last decade, there have been increased efforts to develop new vaccines that can be administered intranasally.

There are several different types of antigens that can induce protective immune responses when administered intranasally, including attenuated organisms, inactivated organisms and acellular subunit vaccines, each having advantages and disadvantages. Attenuated pathogenic microbes, for example, will usually induce protective immunity, but at the same time, there can be significant safety concerns with the use of such a vaccine, such as the potential to cause disease in an immunocompromised host. Furthermore, protection induced against the fully virulent strain may not be complete. An alternative is the generation of killed or inactivated vaccine. The process of inactivation, however, causes many antigens to lose immunogenicity, resulting in poorly protective immune responses. In addition, such vaccines typically need to be administered in the presence of an adjuvant to enhance immunogenicity. There is also the risk that the procedures used are insufficient to completely inactivate the pathogen. Another alternative, therefore, is to use just a portion of the pathogen instead, i.e., a subunit vaccine. As with inactivated pathogens, an adjuvant is usually needed to augment the immunogenicity of a subunit vaccine, but many of the safety concerns surrounding the use of an inactivated pathogen become irrelevant (discussed in Ref. 2).

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Our main objective is to review the recent progress in developing intranasal vaccines. The first subject that is discussed is the use of adjuvants and delivery strategies to enhance the efficacy of intranasal vaccination. Recent advances in the generation of nasal vaccines are then discussed, with particular emphasis on respiratory pathogens that are serious threats to public health, including influenza virus, *Streptococcus pneumoniae* and *Bordetella pertussis.* These organisms are specifically included because either an intranasal vaccine has been generated or there is much promise in developing such a vaccine. Also reviewed is the progress in developing vaccines against pathogens that pose serious threats as potential bioweapons, including *Francisella tularensis, Yersinia pestis* and *Bacillus anthracis.* Finally, there is a discussion of possible intranasal vaccination against sexually transmitted pathogens, including herpes simplex virus type 2 (HSV-2, human herpesvirus 2, HHV-2), *Neisseria gonorrhoeae* and HIV-1. The overall findings to date are summarized in Table I.

MUCOSAL IMMUNITY AND NASAL VACCINATION

The mucosal surface of an adult human covers approximately 400 m^2, including the respiratory, gastrointestinal, conjunctival and urogenital tracts. The mucosa consists of an epithelial monolayer that separates the host from the environment (including commensal and potentially pathogenic organisms) (reviewed in Ref. 3). Underlying this epithelial barrier is the lamina propria, which includes a complex network of mucosal-associated lymphoid tissue (MALT) that is responsible for sampling antigen from the epithelial surface. This immune inductive tissue resembles lymph nodes in structure and contains B-cell follicles, T-cell zones and numerous antigen-presenting cells (APCs), such as macrophages and dendritic cells (4). Although there is some compartmentalization, including different cues for lymphocyte homing to distinct mucosal sites, there is also considerable cross-talk between mucosal and systemic immune responses. Dendritic cells or other APCs that sample antigen at the mucosal surface can migrate to draining lymph nodes and imprint mucosal homing signals on activated lymphocytes. A combination of APC activation, antigen receptor signaling, homing signals and mucosal-specific cytokine/chemokine signals (i.e., retinoic acid, tumor necrosis factor ligand superfamily member 13 [a proliferation inducing ligand, APRIL], tumor necrosis factor ligand superfamily member 13B [B-cell-activating factor, BAFF] and transforming growth factor beta [TGF-β]) preferentially enhances class switching to IgA, a key Ig isotype that is secreted into mucosal tissues and is the first line of defense against pathogen entry (4, 5). Long-lived plasma cells that secrete IgG antibodies reside in peripheral lymphoid tissues (spleen and lymph nodes) and bone marrow, whereas plasma cells secreting IgA and IgM primarily take residence in the gut-associated lymphoid tissue (GALT) lamina propria. Therefore, a distinct advantage of vaccination via the intranasal route is that this procedure can induce both systemic immune responses and responses at multiple mucosal tissues that are potential entry sites for pathogenic microbes, including the urogenital tract (6-9).

Two important components of MALT are Peyer's patches, which are located in the small intestine, and nasopharnyx-associated lymphoid tissue (NALT), which is located in the nasopharyngeal duct of rodents and is regarded as being analogous to Waldeyer's ring in humans (10-12). Both are structurally similar with regard to the presence of M cells, the distribution of APCs (macrophages and dendritic cells), T- and B-cell zones and germinal center formation following antigen delivery. Both T-dependent and -independent IgA class switching have been demonstrated in Peyer's patches and NALT, contributing to IgA in secretions and the induction of an effective mucosal immune response. However, these structures differ in both their times of development and the nature of the immune responses initiated within them. While the development of Peyer's patches begins during embryonic development, the development of NALT occurs postnatally and is triggered by the presence of environmental antigens (11, 13). The targeting of immunogens to Peyer's patches tends to induce immune responses limited to the gastrointestinal mucosa. In contrast, the introduction of antigens into the NALT tends to induce immune responses in the respiratory and reproductive systems (14). The delivery of antigens to NALT via intranasal immunization has been demonstrated to induce both local and systemic immune responses, and thus is a promising vaccination site (11, 13, 15).

ADJUVANTS AND ANTIGEN DELIVERY

Many of the antigens that would be considered for vaccine use, especially subunit and killed/inactivated pathogens, elicit poor protective responses when administered alone intranasally or at other mucosal surfaces (i.e., urogenital or gastrointestinal tracts) (16, 17). In animal models, the use of adjuvants has increased the immunogenicity of these antigens (2).

Adjuvants derived from either toxins or cellular components of bacteria have been demonstrated to be effective in enhancing mucosal immune responses. Cholera toxin (CT) and heat-labile *Escherichia coli* enterotoxin (LT) are two examples of such adjuvants that have been used in animal models. However, toxicity is a problem for human use; therefore, less toxinogenic mutants or derivatives have been generated (18, 19). In the early 1990s, it was discovered that bacterial DNA itself could induce proliferation of lymphocytes (20), and it was later revealed that this effect was due to unmethylated CpG repeats present within the bacterial DNA (21). Synthetic oligonucleotides containing the CpG motif have subsequently been found in animal models to not only augment immune responses generated against intranasally administered antigens, but also to induce protection against specific respiratory pathogens, including *S. pneumoniae* and *Pseudomonas aeruginosa* (22, 23). CpG has been determined to be safe and is currently being tested in numerous clinical trials (24, 25).

The intranasal administration of specific cytokines in conjunction with vaccines has also been demonstrated to increase immunogenicity. Perhaps one of the most successful uses of a cytokine as an adjuvant is interleukin-12 (IL-12). IL-12 has been shown to enhance protective respiratory responses against influenza virus, pneumococcus and *F. tularensis* (26). Although IL-12 was found in human trials to be toxic when administered intraperitoneally (i.p.) or subcutaneously (s.c.), subsequent murine studies revealed that toxicity was greatly reduced when inoculated intranasally, suggesting that IL-12 may be a safe vaccine adjuvant in humans if given via this route (27, 28). Other cytokines that have been demonstrated to be effective adjuvants include IL-1, RANTES and type 1 interferon (29-31).

Another factor that must be considered when attempting to maximize the immunogenicity of an antigen is the vehicle that is used for

Table I. *Summary of successful intranasal vaccine strategies against specific pathogens discussed in the review.*

Pathogen	Intranasal vaccine antigens	Adjuvants/delivery	Animal models demonstrating effectiveness	Current clinical trials	Human vaccine available	Ref.
Influenza	Live, attenuated cold-adapted virus	–	Mouse	Ongoing	FluMist®	33, 39-41
	Inactivated virus	IL-12, LT	Mouse		Switzerland - subunit vaccine	41
	Subunit H1 and N1	IL-12	Mouse		(no longer available)	31, 41, 44, 45
Pneumococcus	Conjugate	IL-12	Mouse			55-58
	Subunit PspA	IL-12	Mouse			59-64
	Unencapsulated inactivated bacteria	–	Mouse			65, 66
B. pertussis	Attenuated organism (gene knockout)		Mouse	Phase I		71-73
F. tularensis	Attenuated organisms (LVS, *capB, SodB*$_{ft}$)	–	Mouse			82-84
	Inactivated organisms	IL-12, CT, Ab/immune complexes	Mouse			85-87
Y. pestis	Attenuated organisms *(EV76, D27-pLpxL)*	Nanoparticles	Mouse			99-101
	Subunit (F1 and V antigens)		Mouse Non-human primates			93-98
B. anthracis	Subunit (PA protective antigen)	Freeze-dried powder, CPG chitosan	Mouse Rabbit			107-112
Herpes simplex	Subunit (glycoprotein D; glycoprotein B)	CpG, viral vector fusion with IgG Fc receptor	Mouse			117, 118
N. gonorrhoeae	Subunit (mixed proteins)	–	Mouse			125, 126
C. trachomatis	Live organism (sublethal)	–	Mouse			123
	subunit					122, 124
HIV-1	Peptide epitope from V3 loop of gp120	BCG	Mouse	Phase I		129
	Subunit (gp41, mixed glycoproteins/proteins)	Virosomes	Non-human primates			127

delivery to the mucosal immune system. The immunogenicity of a soluble antigen can be greatly increased if it is encapsulated within polylactic acid, polylactide-co-glycolide or polyanhydride micro- or nanoparticles. The size and chemical structure of these biodegradable polymer microspheres can be used to control the rate at which the antigen is released, resulting in highly effective priming of the immune response. Liposomes represent another vehicle of interest for mucosal vaccination. These are vesicle-like structures, often composed of phospholipid bilayers, in which the vaccine antigen has been incorporated. The ease of liposome phagocytosis is thought to enhance uptake of antigen by the APCs, which thereby enhances presentation to lymphocytes (17, 32). Immune-stimulating complexes, or ISCOMs, represent a third vehicle that shows promise for intranasal delivery of antigens. Formed from lipids and saponin, these cage-like particles present the antigen to the immune system in a multimeric fashion (32). The complexes are highly immunogenic and are known to stimulate both local and systemic immunity when administered intranasally.

PROTECTION AGAINST VIRULENT RESPIRATORY PATHOGENS

Influenza

The effectiveness of vaccination to prevent influenza infection has been clearly demonstrated (33). There have been at least 4 pandemics of highly virulent influenza within the last 100 years in the U.S., which together have been responsible for well over 600,000 deaths. The most recent 2009 pandemic, also known as the "swine flu" pandemic, serves to remind us not only how deadly the virus infection can be, but also the continuing need for protective vaccines against newly emerging strains (34). The segmented genome of the virus allows it to undergo genetic reassortment when multiple viral subtypes infect the same cell, which can result in the production of virus with a "mixed" genome and altered antigenic determinants. The resulting diversity of new virions can require the development of a new seasonal flu vaccine each year (35). Another complication of influenza infection is heightened susceptibility to secondary bacterial pneumonia. Interferon-gamma (IFN-γ) produced by T cells within the lung during recovery from influenza infection inhibits the ability of alveolar macrophages to phagocytose and clear certain respiratory bacteria, such as *S. pneumoniae*. Indeed, it is believed that over 90% of the deaths that occurred during the 1918 influenza pandemic were due to secondary infection with pneumococcus (36).

Both humoral and cellular branches of the immune system contribute to protective responses against influenza. Antibodies specific for viral surface proteins are able to neutralize the virus and prevent infection of host cells. Antibody-mediated protection is therefore highly effective and is able to prevent subsequent reinfection of the host with the same strain of virus, or a strain that shares antigenic determinants. T-cell-mediated immune responses are also critical for controlling dissemination within the host and resolution of influenza infection. Specifically, cellular immune responses mediated by $CD8^+$ T cells are believed to prevent the spread of influenza virus by direct killing of virally infected cells (37, 38). A vaccine designed to stimulate both branches of the immune system would therefore be ideal.

The standard influenza vaccine is administered by i.m. injection, but this has limited efficacy, in addition to the problems associated with the use of needle-based vaccines, including painful administration and increased risk of infection. In the mid-2000s, an intranasal influenza vaccine became available that consists of a cold-adapted, attenuated virus preparation (39). This vaccine, marketed as FluMist® and manufactured by MedImmune (www.flumist.com), is currently the only intranasally administered vaccine available in the U.S. (33). Recent animal studies with this seasonal vaccine demonstrated that intranasal administration into mice induced effective T-cell responses that not only protected from seasonal influenza but also H1N1 pandemic influenza. Furthermore, intranasal FluMist® vaccination induced resistance to secondary *S. pneumoniae* and *Staphylococcus aureus* challenge, demonstrating that vaccination could prevent inactivation of respiratory antibacterial innate immunity (40).

Despite the success of FluMist®, there are safety concerns associated with its use. Vaccination has been reported to cause cold-like symptoms in some cases, including sore throat and fever, which limits the population approved to receive this vaccine (infants, the elderly and immunocompromised individuals are excluded, the three groups that would benefit most from vaccination) (41). Promising alternatives include the use of inactivated virus or a subunit vaccine, both of which have been shown to have greater efficacy in mouse models when administered intranasally as opposed to subcutaneously (41-43). Inactivated influenza vaccines are currently used in the U.S., albeit by injection but not intranasally (41). An intranasal subunit vaccine became available in Europe in 2001, but the LT used as adjuvant was linked to several cases of Bell's palsy, calling into question the safety of the procedure (44). In spite of this failure, the intranasal administration of a subunit vaccine could be a viable approach provided the proper adjuvant is used. IL-12, for example, which has been shown to be safe when administered intranasally to mice, significantly increases protective humoral immunity induced by a subunit vaccine consisting of hemagglutinin and neuraminidase viral proteins (45). Type 1 interferon, a cytokine that enhances innate immune responses and aids in the priming of primary B-cell responses, has also been demonstrated to be an effective adjuvant for influenza subunit vaccines in mice (31). These studies, as well as others, have demonstrated that intranasal subunit vaccines can be an effective means for preventing influenza infection if the appropriate adjuvant is used.

S. pneumoniae

S. pneumoniae is a Gram-positive bacterium that normally colonizes the mucosal lining of the human nasopharyx. In this location, the pneumococcus acts as a commensal bacterium and is essentially innocuous to the host. However, the bacterium can become highly pathogenic if it colonizes other (normally sterile) sites, including the lung, inner ear and the blood, where it causes pneumonia, otitis media and septicemia, respectively (46). Pneumococcal-associated diseases occur mostly in newborn infants, the elderly and the immunocompromised, and are a leading cause of community-acquired pneumonia (47). The rise of antibiotic-resistant pneumococcal strains and the emergence of a large number of pathogenic strains or serotypes make this pathogen particularly dangerous and a challenge for effective vaccination (46, 48).

Currently, there are two pneumococcal vaccines available in the U.S., both of which are administered by i.m. injection. The 23-valent

pneumococcal polysaccharide vaccine (which is prepared from the purified capsular polysaccharides obtained from 23 different pneumococcal serotypes) can reduce bacteremic levels and improve the prognosis of adults that contract pneumococcal respiratory infections, but it induces poor protective responses in infants and the elderly. The vaccine also loses effectiveness in a relatively short time, requiring revaccination every 5-6 years (49). A newer alternative is the 13-valent conjugate vaccine, containing purified polysaccharides from 13 different pneumococcal serotypes that have been conjugated to diphtheria toxoid protein, which greatly enhances the immunogenicity of the carbohydrate antigens, particularly in the very young (50). This vaccine has been approved for use in children under 2 years old, and has significantly decreased the incidence of invasive pneumococcal diseases in this population. The conjugate vaccine has also been demonstrated to be safe and effective, and to have increased efficacy in the elderly. Unfortunately, while conjugate vaccines are effective against respiratory infections, they appear to have limited efficacy against pneumococcal-induced otitis media (51-53). Another major problem with the use of any polysaccharide-based pneumococcal vaccine is that effectiveness is limited by the small number of serotypes included in the vaccine, such that serotype replacement is now being observed. Alternate methods of vaccination that protect against a broad spectrum of serotypes are being actively investigated (49, 54).

The intranasal administration of pneumococcal conjugate vaccine in conjunction with IL-12 as an adjuvant has been found to induce both systemic and mucosal immune responses in mice, and is protective not only against pulmonary pneumococcal infections, but also against otitis media (55-58). However, this strategy is still limited to the serotypes that are included in the conjugate vaccine. Therefore, a current focus of newer vaccination strategies involves immunization with antigens that are common to all serotypes. Pneumococcal surface protein A (PspA), which is expressed in multiple clinically relevant pneumococcal serotypes, is highly immunogenic, induces cross-reactive responses against multiple pneumococcal serotypes, is able to protect mice from lethal bacterial challenge, and was found in phase I clinical trials to induce the production of pneumococcal-specific antibodies in humans (59-61). Subsequent work has demonstrated that intranasal administration of PspA into mice together with the proper adjuvant (such as IL-12) not only elicits significant antibody-mediated protection, but also prevents heightened susceptibility to secondary pneumococcal challenge after influenza infection (62-64). While much success has been achieved in inducing protective responses in mice using PspA as a vaccine, other antigens and methods of delivery are currently being examined. For example, an alternative to polysaccharide conjugate and protein-based vaccines is the use of whole, killed, unencapsulated pneumococci, which protects laboratory animals against multiple serotypes when administered intranasally (65, 66). Although no intranasal vaccine against pneumococcus is currently available, the success that has been achieved in animal studies using a variety of different antigens suggests that such a vaccine will be available in the near future.

B. pertussis

Whooping cough, which is caused by the pathogen *B. pertussis*, is an example of a disease that has been successfully controlled by vaccination during the latter half of the 20th century, using either whole cell (inactivated) or acellular vaccines. Despite this success, there has been a recent resurgence in the number of pertussis cases, and currently, *B. pertussis* infection accounts for approximately 300,000 deaths per year worldwide. Potential explanations for this resurgence include waning protection in an aging population, increased number of patients opting out of childhood vaccination regimens and the emergence of new virulent strains. Severe reactions to injected inactivated or whole cell pertussis, including swelling and redness at the injection site and high fever, are a major reason for failure to complete the vaccine schedule and have prompted a switch to an acellular vaccine antigen (67). In any case, it has become clear that a new vaccine and vaccination strategies are warranted (67-70).

Experimental studies have demonstrated that a single intranasal administration of a novel attenuated *B. pertussis*, which has mutations in three genes encoding virulence factors, can protect infant mice against challenge with virulent bacteria (71). Subsequent work testing the safety and efficacy of this attenuated mutant in animal models not only revealed that the mutations are genetically stable, but that the vaccine candidate does not cause disease in immunocompromised mice, i.e., IFN-γ receptor knockout mice (72, 73). The success in these animal studies has led to a phase I clinical trial assessing the safety of this intranasally administered strain of *B. pertussis* in humans (ClinicalTrials.gov Identifier NCT01188512). Thus, a nasal vaccine comprised of attenuated strains of *B. pertussis* may soon be available.

PROTECTION AGAINST SELECT AGENTS

F. tularensis

F. tularensis is a highly virulent Gram-negative bacterium that causes respiratory tularemia, a deadly pneumonic disease that can have a 50% mortality rate if left untreated (74, 75). Although the incidence of this disease is rare in the U.S., it has the potential to be used as a bioterrorism agent and the Centers for Disease Control (CDC) has therefore classified it as a Category A select agent (74, 76-78). Efforts to develop a vaccine against this pathogen are discussed, not only because of the highly dangerous nature of this microbe, but also as an example regarding the importance of using proper adjuvants and methods of delivery.

The live vaccine strain of *F. tularensis* (LVS), although attenuated in humans, is not completely effective at inducing protection when administered by scarification or aerosol inoculation, and has been known to cause disease symptoms in immunocompromised individuals; therefore, LVS has not been approved for use as a vaccine in the U.S. (79). The administration of a sublethal dose of *F. tularensis* LVS is able to protect some, but not all, mouse strains from subsequent respiratory challenge with a fully virulent bacterial strain. In addition, induction of protection is dependent upon the route of administration. When LVS is given s.c. or intradermally, no protection against pulmonary infection with a fully virulent bacterium is observed. However, if an aerosol or intranasal inoculum of LVS is administered, protection against virulent challenge can be achieved, indicating that a mucosal immune response must be induced for optimal efficacy (80, 81).

The majority of recent work has focused on developing an effective *F. tularensis* vaccine that lacks the safety issues associated with LVS. One method that has been extensively explored is further genetic attenuation of *F. tularensis* LVS, which has resulted in the successful generation of *capB* and $SodB_{ft}$ mutants (82-84). While both mutants are able to induce protective responses in mice against pulmonary challenge with a fully virulent bacterial strain (SchuS4), the $SodB_{ft}$ mutant has been found to protect both C57BL/6 and BALB/c mice; most other attenuated vaccine candidates, including LVS, have only been demonstrated to protect BALB/c mice (81, 82). The protective responses generated by the $SodB_{ft}$ mutant may therefore be more appropriate for a genetically diverse human population.

Another avenue that is currently being explored is the use of *F. tularensis*-inactivated organisms. Protection against respiratory challenge with live *F. tularensis* LVS can be induced in mice through intranasal vaccination with inactivated, i.e., UV-fixed, bacteria, but the presence of an adjuvant is required for immunization to be maximally effective. Both IL-12 and cholera toxin B (CTB) have been reported to be effective adjuvants for inactivated LVS vaccines and CTB was also able to induce protective responses against SchuS4 (85, 86). Another method for enhancing the immunogenicity of inactivated *Francisella* is the direct targeting to Fc receptors on host APCs through opsonization of IgG_{2a} anti-*F. tularensis*-coated bacteria. Intranasal administration of such antibody–antigen complexes not only protects mice against subsequent challenge with *F. tularensis* LVS, but can also partially protect against respiratory challenge with the highly virulent SchuS4 strain (87). These experiments demonstrate proof of principle that vaccination through the intranasal route is not only a viable approach for protection against pulmonary tularemia, but may be a highly effective method of inducing protective responses against various potential bioweapons.

Y. pestis

Y. pestis is a Gram-negative bacterium and the causative agent of plague. Pneumonic plague, which results from a respiratory infection with the pathogen, is the most severe form of the disease because of its rapid onset, the ease at which it can be spread from person to person, and the high mortality rate (near 100% if untreated). Although *Y. pestis* was responsible for severe pandemics throughout history, currently, cases of plague are relatively few, and smaller-scale infections are a continuing public health problem for developing countries. A current major concern, however, is the potential for *Y. pestis*, like *F. tularensis*, to be used as a bioweapon; therefore, an effective vaccine against this pathogen is being sought (88, 89).

Animal studies have shown that protective immunity against *Y. pestis* can be mediated by either the humoral or cellular branches of the immune system, thus potentially allowing for the development of several diverse yet effective immunization strategies (88, 90-92). One vaccination approach that has shown promise in animal models is the administration of a subunit vaccine consisting of the *Y. pestis* F1 and V antigens (93-95). While this strategy has been shown to be effective in mice, it was not protective in certain species of nonhuman primates; therefore, further research is needed to find ways to increase the protective response induced by these antigens (88).

The use of micro- and nanoparticles to deliver specific antigens to the immune system is a technique that has been extensively studied in the pursuit of generating an effective vaccine against *Y. pestis*. Eyles et al. found that mice that were intranasally immunized with polylactic microspheres containing the F1 and V antigens (in the absence of an adjuvant) were partially protected against aerosol challenge with *Y. pestis*, while mice that received these antigens in soluble form were not protected at all, demonstrating that the immunogenicity of soluble antigens can be increased by this method of delivery (96). Different variations in the construction and administration of these synthetic capsules have been examined over the years in order to further increase the vaccination efficacy of the molecules contained within. Currently, there are at least two studies that report the induction of long-term protection against *Y. pestis* in mice from a single intranasal immunization with nanosphere-encapsulated protein (97, 98).

Even with techniques to increase their immunogenicity, another problem that must be considered when using the F1 and V antigens as vaccine candidates is that not all virulent strains of *Y. pestis* express F1 and there is variation in the V antigen, suggesting that other antigens should be considered for vaccine use. An alternative to the use of the F1-V subunit vaccine is the use of live, attenuated *Y. pestis* strains, such as EV76, which was one of the earliest attenuated strains to be used for vaccination. Although EV76 has been shown to be effective in humans, it is still reactogenic and can cause chronic infections, limiting it use worldwide (99); therefore, throughout the last decade, much work has been dedicated to studying or generating other attenuated strains of *Y. pestis* as vaccine candidates (88, 90, 92). An effective method that has recently been employed to further attenuate *Y. pestis* strains is the expression of the *E. coli lpxl* gene in *Y. pestis*, which results in the production of lipopolysaccharide molecules that interact with and stimulate Toll-like receptor 4. This not only decreases the virulence of the bacteria, but also increases its immunogenicity, making it a safe and effective vaccine candidate when administered intranasally to laboratory animals (99-101).

B. anthracis

B. anthracis, the causative agent of anthrax, is another example of a pathogen that is no longer a severe public health problem due to effective preventive and eradication measures, but as recent history has demonstrated, it still poses a serious threat as a bioweapon. This Gram-positive bacterium, like *F. tularensis* and *Y. pestis*, can infect a host via multiple sites, but it is most deadly when it infects the respiratory tract. *B. anthracis* is able to form dormant spores that are resistant to both extreme environmental/man-made conditions and time (dormant spores can survive for decades), making their dissemination as a bioweapon potentially easy (102-104).

As mentioned above, preventive measures were taken to control outbreaks and the spread of anthrax; therefore, an effective vaccine was developed in both humans and livestock. AVA (Anthrax Vaccine Adsorbed), or BioThrax®, the only FDA-licensed human anthrax vaccine in the U.S., is comprised of filter-sterilized culture supernatant from an attenuated, nonencapsulated strain of the bacterium and aluminum hydroxide gel (as an adjuvant) (discussed in Refs. 104, 105). Although effective, the immunization schedule involves a grueling regimen of multiple s.c. injections at different intervals over a period of 6 weeks. In addition, laboratory studies have shown that

this vaccine does not protect all tested animal species equally, and that antibody responses generated in humans are variable (discussed in Refs. 104, 106). Most of the effective antibodies generated from AVA vaccination are against a protein dubbed "protective antigen", or PA; therefore, attempts to generate subunit vaccines using a recombinant version of this protein have been undertaken. Several studies with laboratory animals have demonstrated that intranasal immunization with recombinant PA (with a variety of different adjuvants) can induce protective responses against challenge with lethal *B. anthracis* (107-109). During the past few years, powdered freeze-dried formulations of this antigen were developed that were not only stable during storage (with increased shelf-life), but, when given intranasally, effectively protected rabbits from lethal anthrax challenge (110-112). While no intranasal vaccine against this pathogen has yet been tested in humans, the encouraging results of these animal studies suggest that a less burdensome yet effective vaccine may soon be available.

PROTECTION AGAINST SEXUALLY TRANSMITTED DISEASES

An advantage of intranasal immunization is that it utilizes the common mucosal system and can elicit protective immunity in all mucosal surfaces, including the urogenital tract (113). Therefore, intranasal vaccines can potentially be used to prevent the spread of sexually transmitted diseases, including herpes, bacterial infections, and even HIV.

Genital herpes, which is caused by either HSV-1 or HSV-2, accounts for one of the most commonly encountered sexually transmitted diseases worldwide. Although HSV-2-specific antibodies can contribute to protective responses against the virus, the bulk of the protection appears to be mediated by IFN-γ-producing $CD4^+$ T cells (114). In spite of this knowledge, there is currently no herpes vaccine available, and although progress has been made in treating herpes infection, there is still no cure. After the failure of Herpevac™ (GlaxoSmithKline) to make it through clinical trials (www.niaid.nih.gov/news/QA/pages/HerpevacQA.aspx; www.clinicaltrials.gov), new vaccine strategies against this virus are warranted (discussed in Refs. 115, 116). Herpevac™, which contains HSV-2 glycoprotein D, like most vaccines, was administered by injection. Research with laboratory animals has demonstrated, however, that intranasal immunization with a viral vector expressing another glycoprotein, glycoprotein B, induced strong protective responses against HSV-2, which were longer-lived and more robust than those induced when this same vector was injected i.p. (117). A more recent and novel study, in which a fusion protein consisting of glycoprotein D and the mouse IgG Fc fragment was administered intranasally to mice in conjunction with CpG, further demonstrated the effectiveness of the use of an intranasal inoculation route (118), giving credence to the idea that intranasal vaccination with glycoprotein D or another HSV-2 antigen may be an effective method for preventing herpes infections in humans.

In addition to viruses, a myriad of bacterial infections can be transmitted through sexual contact, two of the most common being *Chlamydia trachomatis* and *N. gonorrhoeae* (119, 120). Both *C. trachomatis* and *N. gonorrhoeae* are intracellular pathogens that present unique challenges for developing protective immune responses. As with many respiratory pathogens, antibodies that react with *N. gonorrhoeae* attachment pili are protective for preventing infection; however, a high rate of antigenic variation limits the effectiveness of these antibodies (121). Immunity to *C. trachomatis* is not as well defined. The obligate intracellular lifestyle of C. *trachomatis* limits antibody accessibility, and cytotoxic T-cell immunity against the pathogen is poor due to a lack of presentation of its proteins on MHC class 1 molecules (121). Several studies have demonstrated that intranasal inoculation of mice with either live *C. trachomatis* or with *Chlamydia*-derived proteins was able to offer some degree of protection against vaginal challenge with the bacterium, resulting in reduced bacterial loads and shorter durations of infection (122-124). Other animal studies using outer membrane preparations or proteins derived from *N. gonorrhoeae* have produced similar findings (125, 126). Although many of these studies used adjuvants not currently approved for human use, including CTB, they demonstrated proof of principle for a common mucosal system and that intranasal vaccines against sexually transmitted infection show promise.

HIV-1 infection is spread primarily though sexual contact, and over 30 million people are currently infected worldwide (115, 127). Attempts to generate an effective HIV vaccine have been challenging, however, largely due to the virus's ability to evade humoral immunity because of its ever-changing viral envelope, and to date there is no vaccine available (115). In spite of this, it has recently been suggested that the induction of both humoral and cell-mediated immune responses is critical for combating HIV infection, and that neutralizing antibodies are a key effector mechanism against the virus (128, 129). Additionally, due to the chronic nature of HIV infection, therapeutic vaccines that help clear or reduce the severity of infection are being sought alongside preventive vaccines. Attempts to develop an intranasally deliverable vaccine in animal models date back to the early 1990s (130-133). A potential nasal vaccine candidate was generated in 2001 in the form of a recombinant strain of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) that expressed and secreted a peptide containing a portion of the V3 epitope present within gp120 (rBCG-V3J1). The inoculation of rBCG-V3J1 into mice deficient in IL-4 and IFN-γ, which somewhat mimics the immunodeficiency induced in humans by HIV infection, was able to induce the production of HIV-neutralizing antibodies, suggesting that this vaccine will be therapeutic as well as prophylactic (129).

Despite the success in animal models, human clinical trials with nasal vaccine candidates have yielded disappointing results. For example, a recent phase I clinical trial evaluating the intranasal inoculation of a recombinant gp160 protein revealed that, while it was well tolerated, it induced poor antibody responses (134). Despite this setback, efforts are still being undertaken to generate an effective vaccine against HIV. Bomsel et al. recently showed that the combined intranasal and i.m. inoculation of rhesus macaques with virosomes (similar to liposomes but containing viral glycoproteins) containing the HIV-1 protein gp41 was able to protect against vaginal challenge with simian HIV (135). In 2009, a phase I clinical trial began to examine the safety as well as the magnitude of the immune responses generated by using a similar vaccination strategy, in which combined intranasal and i.m. immunizations of virosomes containing HIV peptides were administered to patients (www.clinicaltrials.gov). While it will take time before the results of this study are known, its execution reflects the hope that an intranasal vaccine against this virus may serve as a means for controlling the spread of infection.

FUTURE PERSPECTIVES

As of today, FluMist® is the only intranasal vaccine that is available for commercial use in the U.S. While there are some drawbacks, its success may pave the way for the approval of other intranasal vaccines. An intranasal vaccine is needed for public health pathogens such as *S. pneumoniae,* which routinely causes illness and deaths in millions of Americans per year, mostly in infants or the elderly (47, 136). In addition to being administered without the use of needles, which can potentially aid the spread of other infectious diseases, intranasal vaccinations have been shown in many cases to be more effective than those given by parenteral routes. Currently, intranasal vaccination against *S. pneumoniae* and other pathogens has been successful in laboratory studies, but has not been extensively tested in humans. Indeed, there are only two phase I clinical trials looking at the safety of intranasal vaccines against HIV and *B. pertussis.* Despite this conspicuous absence of clinical trials examining this method of vaccination, the success that has been met in animal models strongly suggests that the value of intranasal vaccination will be increasingly recognized in the future.

DISCLOSURES

The authors state no conflicts of interest.

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